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RNAi of the circadian clock gene *period* disrupts the circadian rhythm but not the circatidal rhythm in the mangrove cricket

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The clock mechanism for circatidal rhythm has long been controversial, and its molecular basis is completely unknown. The mangrove cricket, *Apteronomobius asahinai*, shows two rhythms simultaneously in its locomotor activity; a circatidal rhythm producing active and inactive phases and a circadian rhythm modifying the activity intensity of circatidal active phases. The role of the clock gene *period* (*per*), one of the key components of the circadian clock in insects, was investigated in the circadian and circatidal rhythms of *A. asahinai* using RNAi. After injection of double-stranded RNA of *per*, most crickets did not show the circadian modulation of activity but the circatidal rhythm persisted without a significant difference in the period from controls. Thus, *per* is functionally involved in the circadian rhythm but plays no role, or a less important role, in the circatidal rhythm. We conclude that the circatidal rhythm in *A. asahinai* is controlled by a circatidal clock whose molecular mechanism is different from that of the circadian clock.

Keywords: *Apteronomobius asahinai*; circatidal clock; locomotor activity

1. INTRODUCTION

Circatidal rhythms are endogenous rhythms with a period of approximately 12.4 h corresponding to tidal flooding and ebbing, and have been documented in various organisms, mostly those living in the intertidal zone [1, 2]. The clock mechanism for circatidal rhythms has long been controversial. There are two leading hypotheses: One is that circatidal rhythms are tidally adapted circadian rhythms [3, 4], and the other is that a circatidal clock with a period of approximately 12.4 h produces the circatidal rhythm [5, 6]. The former hypothesis is based on the premise that common molecular components control both the clock producing circatidal rhythms and the circadian clock. The oscillation of the circadian clock is regulated by cyclical expressions of circadian clock genes and proteins through feedback mechanisms [7]. However, the molecular mechanisms generating circatidal rhythms are completely unknown [2, 8].

The mangrove cricket, *Apteronomobius asahinai* is endemic to mangrove forests, and forages on the forest floor during low tide and rests during high tide. Under constant darkness, adults of this species show a clear and persistent circatidal activity rhythm with a free-running period of approximately 12.6 h [9]. Moreover, a circadian rhythm modifying the circatidal rhythm by inhibiting activity during subjective day free-runs under constant darkness and can be entrained by light-dark cycles [9]. In insects the molecular basis of the circadian clock has been extensively studied [10], and the predominant role of the gene *period* (*per*) is confirmed in some species including a cricket, *Gryllus bimaculatus* [11]. Thus, the mangrove cricket is a suitable subject for examining the molecular basis of the circatidal rhythm.

In the present study, we performed RNAi of *per* (*per* RNAi) to examine whether a clock possessing a common molecular basis with the circadian clock is involved in the circatidal rhythm in *A. asahinai*. The results showed that *per* RNAi disrupted the circadian modulation of the activity rhythm but did not affect the circatidal rhythm.

2. MATERIALS AND METHODS

Adults of *A. asahinai* were collected from mangrove swamps in Kin (26°27' N, 127°56' E) and Ginoza (26°30' N, 127°59' E), Okinawa Prefecture, Japan in May-November, 2007-2009. A laboratory culture originating from the collected crickets was kept at $25.0 \pm 1.0^{\circ}\text{C}$ under LD 12:12 (12 h light and 12 h darkness).

Total RNA was isolated from heads using Trizol (Invitrogen). cDNA synthesis and clone development for *per* and *Elongation factor-1 α* (*EF-1 α*) were performed as described in [12]. To obtain the complete sequence of *per*, 5' and 3' RACE (rapid amplification of cDNA ends) were also performed using a SMART RACE cDNA Amplification Kit (Clontech). Nucleotide sequences of *per* and *EF-1 α* were deposited in DDBJ/GenBank/EMBL (AB550300-AB00550302). Double-stranded RNAs (dsRNA) of approximately 500 bp for *per* (*dsper*) and for bacterial β -lactamase (*dsbla*), used as a control, were synthesized using T7 RiboMAX Express Large Scale RNA Production Systems (Promega). Primers are listed in electronic supplementary material, table S1. The dsRNAs were suspended in water and their concentration was adjusted to 0.5 $\mu\text{g}/\mu\text{l}$.

To verify gene silencing, quantitative real-time PCR with standard curve methodology was performed to measure *per* mRNA levels using GoTaq qPCR Master Mix (Promega) according to [12]. Male crickets within 10 days of adult emergence were collected from a laboratory colony, anaesthetized with CO_2 and immediately placed on a slide glass, and injected with 1 μl of *dsper* or *dsbla* solution with a glass capillary into the abdomen. Five days after the treatment, survivors were individually collected at ZT 7, 13 and 19 (ZT stands for Zeitgeber Time, and the onset of light is defined as ZT 0), and total RNAs were extracted from the head without antennae. *EF-1 α* was used as a control gene for normalization.

Forty male crickets collected in the field were used for activity recording from the day or next day following collection. They were individually housed in plastic chambers with unlimited food and water supply. An infrared beam (EE SPW-321, Omron) was set across the

chamber, and the number of interruptions of the beam was recorded at 6 min intervals on a personal computer [13]. The recording chambers were set in an incubator kept at 25°C and equipped with a 15W fluorescent lamp (FL15W, Panasonic) with an irradiance of approximately 1.0-3.5 W·m⁻².

Circatidal rhythmicity was determined using a chi-square periodogram [14]. If there was a single peak between 10 and 15 h in the periodogram above the 0.05 confidence level, the activity was judged to be circatidal. Circadian rhythmicity was determined by comparison of activity levels between the adjacent circatidal cycles. In *A. asahinai*, a circadian rhythm modifies activity levels in circatidal active phases [9]. When a circatidal rhythm was detected, the number of infrared beam interruptions was counted for every circatidal period shown by the chi-square periodogram. The difference in the activity levels counted for each circatidal period of even and odd number cycles was examined by analysis of covariance on the assumption that the activity levels regress linearly. The activity was judged to be influenced by a circadian rhythm when the slope or the elevation was significantly different between even and odd number cycles ($P \leq 0.05$) (electronic supplementary material, figure S1).

3. RESULTS

Quantitative real-time PCR verified that RNAi of *per* is effective for reducing the expression of *per* mRNA and abolishes its temporally variable expression in *A. asahinai* (electronic supplementary material, figure S2).

Locomotor activities were recorded under LD 12:12 for 10 days and under constant darkness for the subsequent 10 days. Crickets were only anaesthetized, injected with *dsbla* or injected with *dsper* at the middle of the photophase on the 6th day. All crickets except an intact one showed a circatidal rhythm irrespective of the treatment, and there was no significant difference in the period among the three groups (figures 1, 2A). In the intact and *dsbla*-injected groups, a circadian rhythm was shown in approximately 90% and 70% crickets, respectively (figures. 1AB, 2B). In the *dsper*-injected group, however, only 1 of 12 crickets

showed a circadian rhythm and there were significant differences in the proportion of crickets with a circadian rhythm between the *dsper*-injected group and the other two groups (figures 1C, 2B).

4. DISCUSSION

The hypothesis proposed by Palmer [1, 3, 4] that circatidal rhythms are tidally adapted circadian rhythms has been supported by the fact that circatidal rhythms are easily changed to circadian rhythms that can be entrained to light-dark cycles in a few species [15, 16]. If this hypothesis is applicable to *A. asahinai*, the circatidal rhythm should be disrupted by knockdown of *per* by RNAi because *per* is one of the key components of insect circadian clocks [10]. However, *per* RNAi disrupted the circadian modulation of activities but did not affect the circatidal rhythm. Thus, this hypothesis is not supported by present evidence.

Naylor [5, 6] proposed the alternative hypothesis that the circatidal clock different from the circadian clock produces the circatidal rhythm. In *A. asahinai* a clear and persistent circatidal activity rhythm is shown and a circadian rhythm modifies the activity independently from the circatidal rhythm [9] as in some other intertidal animals [5, 17, 18]. In these species, therefore, existence of the circatidal clock independent of the circadian clock is probable. In *A. asahinai*, the phase response curve for inundation pulses in the circatidal rhythm resembled that of circadian rhythms for light pulses, suggesting the existence of a circatidal clock with a period approximately half that in circadian clocks [19]. Although the period is different between the circatidal and circadian clocks, the circatidal clock in *A. asahinai* could conceivably be driven by the same molecular mechanism as that of the circadian clock, because the circatidal clock has similar characteristics to a circadian clock in the phase response [19]. If this is the case, then knockdown of the circadian clock gene *per* should disrupt the circatidal rhythm in addition to the circadian modulation of activities. The present results, however, demonstrated that the circatidal rhythm of *A. asahinai* persisted even after

disruption of the circadian modulation of activities by knockdown of *per*. It is, therefore, probable that the circatidal rhythm in *A. asahinai* is controlled by a circatidal clock in which *per* is not involved.

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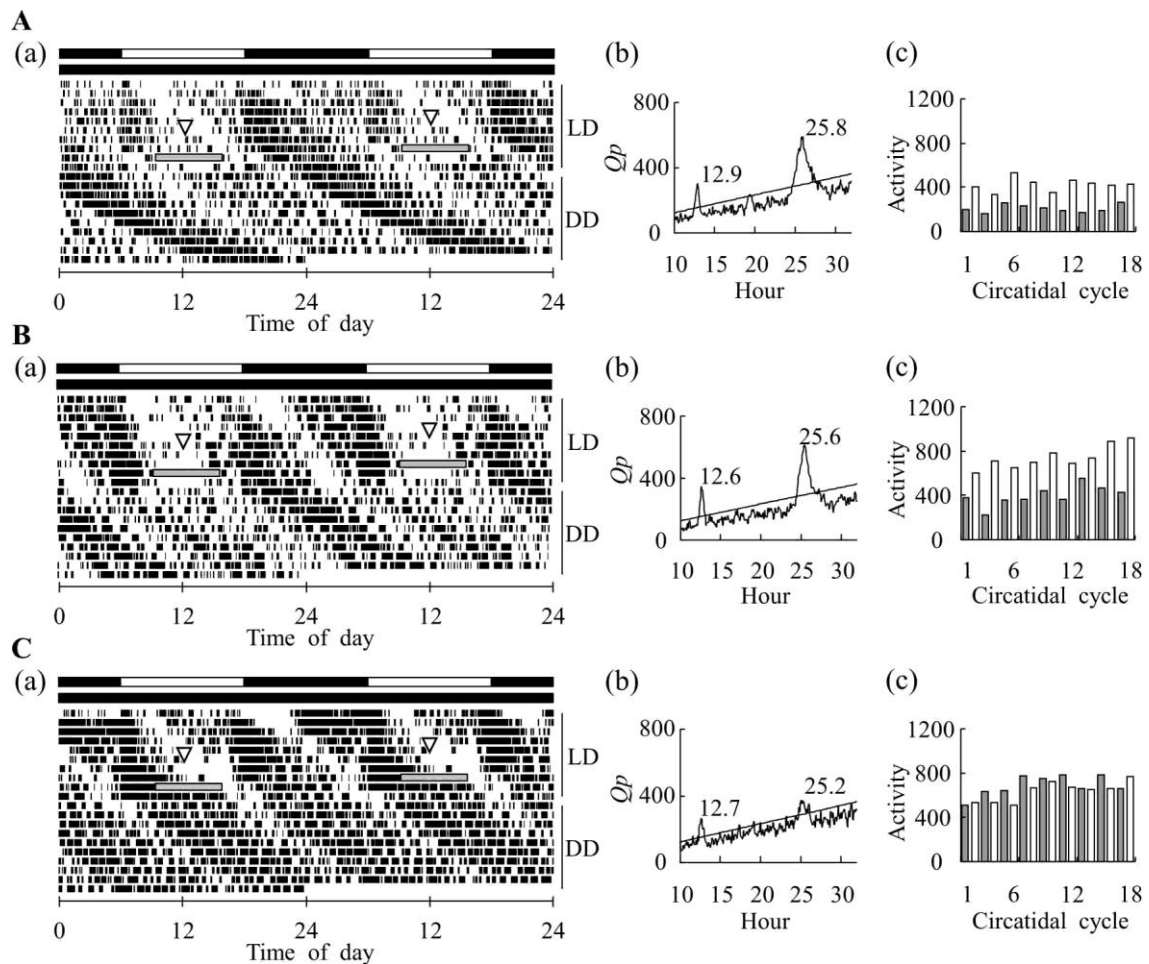


Figure 1. Examples of the locomotor activity rhythm in male adults of *Apteronomobius asahinai* anaesthetized (control) (A), injected with double-stranded RNA for β -lactamase (B), and injected with double-stranded RNA for *per* (C) and transferred from light-dark cycles (LD) to constant darkness (DD). Injection was performed on the 6th day (triangles). Double-plotted actograms (a), chi-square periodograms of activities in subsequent DD (b), and histograms of activity levels counted for each circatidal period in DD (c) are shown. Black and white bars above the actograms indicate light and dark phases, respectively. Gray boxes indicate no data. The oblique line in the periodogram indicates the significance level of $\alpha = 0.05$ and a peak value above the line is designated as significant. Grey and white histograms represent odd and even circatidal cycles, respectively.

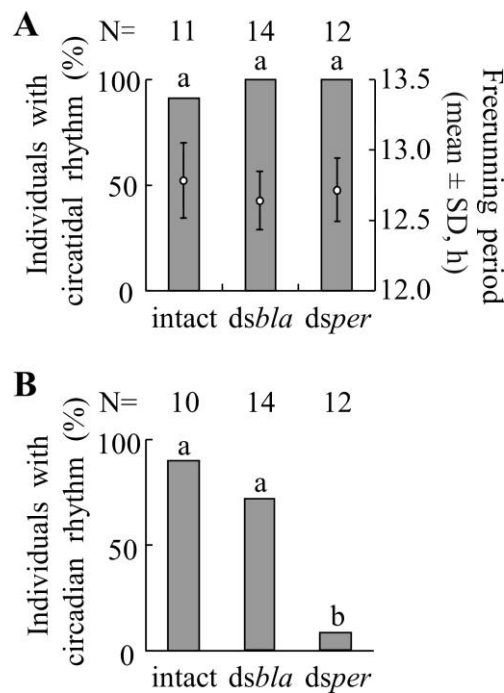


Figure 2. The effects of *per* RNAi on circatidal and circadian rhythms in male adults of *Apteronomobius asahinai* under constant darkness subsequent to light-dark cycles. Crickets were anaesthetized (intact control), injected with double-stranded RNA for β -lactamase (*dsbla*), or injected with double-stranded RNA for *per* (*dsper*). The proportions with the same letters in each panel are not significantly different (Tukey-type multiple comparisons for proportions, $P > 0.05$). (A) the proportion of crickets with a circatidal rhythm and its period determined by a chi-square periodogram. The circatidal periods were not significantly different among the three groups ($P > 0.05$, ANOVA). (B) the proportion of crickets with a circadian rhythm among those with a circatidal rhythm determined by analysis of covariance for the activity levels of even and odd circatidal cycles (electronic supplementary material, figure S1).